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Effect of feeding the ionophores monensin and laidlomycin propionate and the antimicrobial bambermycin to sheep experimentally infected with *E. coli* O157:H7 and *Salmonella typhimurium*[1]

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ABSTRACT: *Escherichia coli* O157:H7 and *Salmonella* are widely recognized as important agents of food-borne disease with worldwide distribution. The use of ionophores in feeding growing ruminants is widespread in the United States and has attracted recent interest due to the apparent temporal relationship between initial ionophore use and the increase in human *E. coli* O157:H7 cases. Two experiments were conducted to evaluate the effects of short-term feeding of ionophores on fecal shedding, intestinal concentrations, and antimicrobial susceptibility of *E. coli* O157:H7 and *S. typhimurium* in growing lambs. Sixteen lambs were used in each experiment, four lambs per treatment group: monensin, laidlomycin propionate, bambermycin, and a control treatment. Lambs were fed a grain and hay (50:50) diet with their respective ionophore for 12 d before experimental inoculation with *E. coli* O157:H7 or *S. typhimurium*. Animals were maintained on their respective diets an additional 12 d, and fecal shedding of inoculated pathogens was monitored daily. Lambs were killed and tissues and contents were sampled from the rumen, cecum, and rectum. No differences ($P > 0.05$) in fecal shedding of *Salmonella* or *E. coli* O157:H7 were observed due to treatment. Occurrence of *Salmonella* or *E. coli* in luminal contents and tissue samples from the rumen, cecum, and rectum did not differ ($P > 0.05$) among treatments. Feeding monensin decreased ($P < 0.05$) the incidence of scours in sheep infected with *Salmonella* compared with the other treatments. No differences in antimicrobial susceptibility were found in any of *Salmonella* or *E. coli* O157:H7 isolates. Results from these studies indicate that short-term ionophore feeding had very limited effects on *E. coli* and *Salmonella* shedding or on antimicrobial susceptibility in experimentally infected lambs.

Key Words: *Escherichia coli* O157:H7, Ionophores, *Salmonella*, Sheep

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Introduction

In the United States, medical and productivity costs associated with bacterial human food-borne illness have been estimated at $2.9 to $6.7 billion per year. Illness caused by *Salmonella* and *E. coli* O157:H7 account for almost half of these costs (Buzby et al., 1996). *Escherichia coli* O157:H7 has been isolated from beef and dairy cattle at all stages of production, and although shedding is intermittent and can be difficult to detect, it appears to be fairly widespread throughout the bovine population (USDA, 1997; Hancock et al., 1998; Elder et al., 2000). *Salmonella* populate the intestinal tracts of various animal species, including beef and dairy cattle, which represent a major reservoir for human food-borne salmonellosis (Fedorka-Cray et al., 1998).

Ionophores were approved by the U.S. FDA in the mid-1970s as feed additives, and since then, their use has become routine in the feeding of growing ruminants. The use of ionophores has attracted interest, given the apparent temporal relationship between initial ionophore use in the U.S. cattle industry and the increase in *E. coli* O157:H7 cases (Griffin and Tauxe, 1991; Rasmussen et al., 1999). Researchers have suggested that because *E. coli* is a gram-negative bacterium, ionophores could increase the incidence of *E. coli* in cattle by inhibiting competing gram-positive species (Dennis et al., 1981; Henderson et al., 1981; Schelling, 1984). However, survey data and experimentation in

[1] Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable.

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cattle have yielded conflicting results (Garber et al., 1995; Dargatz et al., 1997; Herriott et al., 1998). We hypothesize that the ability of ionophores to alter the gut microbiota may give *E. coli* and/or *Salmonella* a selective advantage and warrants research. Therefore, two experiments were conducted to evaluate the effects of feeding the ionophores monensin and laidlomycin propionate and the antibiotic bambermycin on *E. coli* O157:H7 and *Salmonella typhimurium* in experimentally infected sheep.

Materials and Methods

Animals and Experimental Design. For each of two experiments, 16 Suffolk wether and ewe lambs (average BW = 35 kg) were housed indoors in individual pens with ad libitum access to Bermudagrass hay and water. A commercial lamb diet was provided to each lamb starting at 0.2 kg/d increasing over a 10-d period to a final feeding level of 1 kg animal^{-1} d^{-1} (Table 1). Hay intake was slowly decreased over this same time so that lambs were consuming approximately a 50:50 concentrate to hay diet. Lambs were randomly assigned to receive daily in their feed, one of four treatments: 1) control (**CON**): no ionophore; 2) monensin (**MON**): 90 mg animal^{-1} d^{-1}; 3) laidlomycin propionate (**LP**): 37.5 mg animal^{-1} d^{-1}; and 4) bambermycin (**BBM**): 5 mg animal^{-1} d^{-1}. Lambs continued an additional 4 d on full feed before bacterial challenge as described below and remained on this diet throughout the 12-d experimental period. Sheep were killed (Euthasol, Delmarva Laboratories, Inc., Midlothian, VA) on d 12 of each experiment, and tissue from the rumen, cecum, and rectum, as well as their respective lumen contents (10 to 15 g) were aseptically collected for bacterial enumeration as described below. Care was taken to ensure each tissue and lumen content sample was removed from approximately the same location on each animal. Additionally, ileocecal lymph nodes were collected in Exp. 1. The incidence of scours (no indication of fecal pellet formation) was recorded daily in Exp. 1. The Animal Care and Use Committee of the Food and Feed Safety Research Laboratory, USDA preapproved care, use, and handling of experimental animals.

Table 1. Ingredient composition of grain diet fed to lambs (dry matter basis)

Item	%
Cracked corn	43.9
Soybean meal	5.5
Urea	0.4
Trace mineral mix[a]	0.2
Coastal Bermuda hay	50.0

[a]Composition (as-fed basis): protein, 0.26%; fat, 2.0%; Ca, 1.9%; P, 0.03%; S, 1.63%; Mg, 0.02%; Mn, 160,000 ppm; Fe, 11,000 ppm; Cu, 52,000 ppm; Co, 2,400 ppm; Zn, 190,000 ppm; I, 5,500 ppm; and Se, 1,100 ppm.

Bacterial Cultures. Salmonella typhimurium strain NVSL 95-1776, which was naturally resistant to novobiocin, was made resistant to nalidixic acid in our laboratory via successive cultivation in tryptic soy broth (**TSB**) containing up to 20 μg/mL of nalidixic acid. *Escherichia coli* O157:H7 strain BDMS T4169 (ATCC 700728) was obtained from the American Type Culture Collection (Manassas, VA) and was cultivated in anoxic TSB medium at 37°C. This strain was made resistant to novobiocin and nalidixic acid (20 and 25 μg/mL, respectively) via the same procedures as above. These novobiocin/naladixic resistant phenotypes were stable through multiple unselected transfers in batch culture and through repeated culture vessel turnovers in continuous culture (data not shown). Overnight cultures (1,000 mL) were harvested by centrifugation (7,500 × *g*, 10 min) and the cell pellets were resuspended in TSB medium (150 mL total volume). Sheep were individually inoculated with 10 mL of TSB containing *S. typhimurium* (Exp. 1; 1×10^9 cfu) or *E. coli* O157:H7 (Exp. 2; 4×10^{11} cfu) via oral gavage. Fecal samples were collected 3 d prior to dosing and were screened for the presence of wild-type *Salmonella* and *E. coli* O157:H7. The day following dosing (d 1) and on each of the subsequent 11 d, fecal samples were collected and fecal shedding of inoculated *Salmonella* or *E. coli* were enumerated as described below. Qualitative enumeration was conducted daily in both experiments and quantitatively on d 3, 4, 5, 6, 7, 8, 10, 11, and 12 in Exp. 1 and on all 12 d in Exp. 2.

Bacterial Enumeration. Ten to fifteen grams of fecal material was collected from each lamb daily. From each composited fecal sample, 1 g of fecal material was serially diluted (10-fold increments) in sterile PBS and plated on brilliant green agar (for inoculated *Salmonella*) or MacConkey's agar (for inoculated *E. coli* O157:H7); each agar was supplemented with novobiocin (20 μg/mL) and nalidixic acid (25 μg/mL). Plates were incubated 24 h at 37°C and colonies that grew on agar plates were directly counted. In order to qualitatively confirm the presence of inoculated *E. coli* O157:H7 and *S. typhimurium*, daily fecal samples, intestinal contents, and epithelial tissue samples were incubated (24 h, 37°C) in 10 mL of tetrathionate broth (*Salmonella*) or 20 mL of GN Hajna with novobiocin/naladixic acid (*E. coli* O157:H7) and streaked on agar plates as above. Plates showing colony growth were judged to be positive for their respective bacterial species (qualitative enumeration).

Determination of Antimicrobial Susceptibility. Salmonella and *E. coli* isolates were collected on d 1, 6, and 12 of each experiment and examined for antimicrobial susceptibility using the National Antimicrobial Resistance Monitoring System (NARMS) 2001 panel. Minimum inhibitory concentrations (**MIC**) for antimicrobials were determined by broth microdilution according to methods described by the National Committee for Clinical Laboratory Standards (NCCLS, 1999). Susceptibility testing was performed using the Sensititre auto-

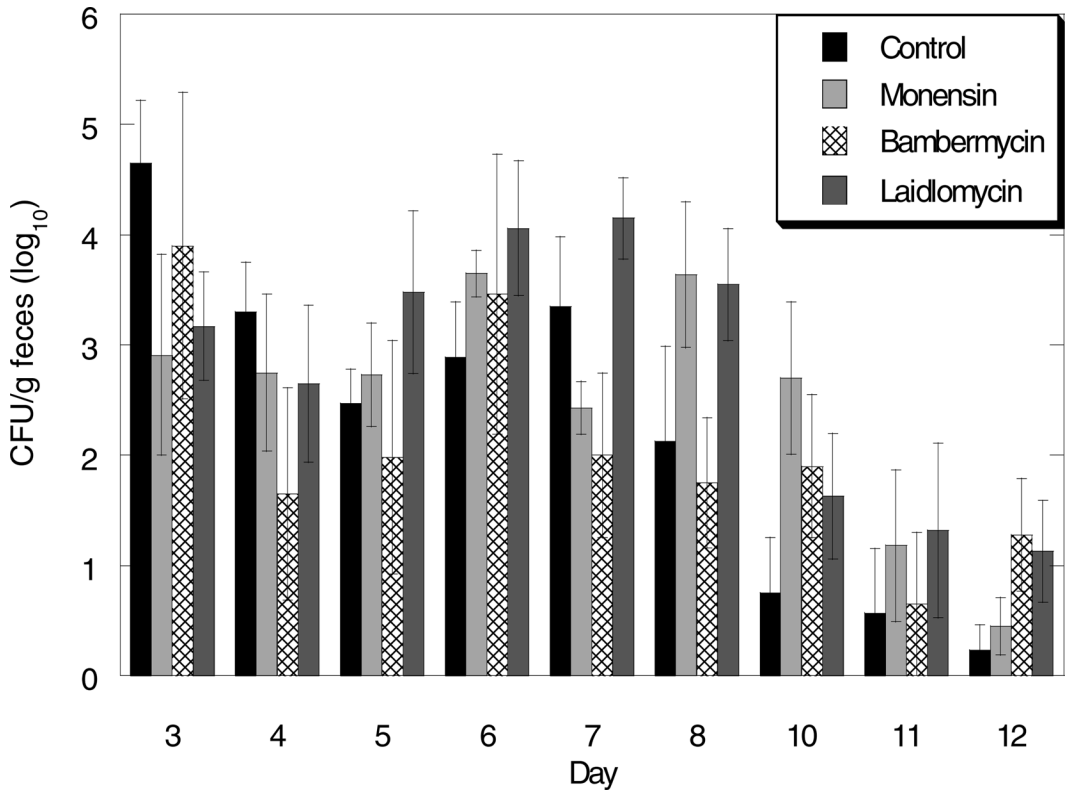

Figure 1. Fecal shedding (cfu/g feces [$\log_{10}$]) of *S. typhimurium* in experimentally infected sheep fed a control diet or diets containing monensin, bambermycin, or laidlomycin propionate.

mated antimicrobial susceptibility system according to the manufacturer's instructions (Trek Diagnostic Systems, Westlake, OH). The following antimicrobials were assayed: amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim/sulfamethoxazole. Resistance breakpoints were determined using NCCLS interpretive standards (NCCLS, 1999) unless unavailable, in which case breakpoints in the NARMS 2000 Annual Report (FDA, 2000) were used. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Enterococcus faecalis* ATCC 29212 were used as quality control strains for broth microdilution susceptibility testing.

Ionophores, Reagents and Supplies. Laidlomycin propionate (Cattlyst) was generously provided by Alpharma Inc. (Chicago Heights, IL) and BBM (Gainpro) was provided by Hoechst Roussel Vet (Warren, NJ). Monensin (Rumensin) was from Elanco (Greenfield, IN). Unless otherwise noted, all media and agar were from Difco Laboratories (Detroit, MI). Reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical Analysis. Data were analyzed using SAS Version 8.02 (SAS Inst., Inc., Cary, NC). Data for daily fecal shedding of bacteria were analyzed using the Proc Mixed procedure with treatment, day, and lamb included in the model and reported as least squares means ± SEM. Logistic regression was used to analyze the incidence of scours and qualitative bacterial enumeration. Bacterial counts from luminal contents (quantitative) were subjected to ANOVA appropriate for a completely randomized design. Differences among means were considered significant at a 5% level of significance. Power analysis was conducted using GPOWER software (Erdfelder et al., 1996).

Results

Experiment 1. Fecal samples collected prior to inoculation with *S. typhimurium* were negative for wild-type *Salmonella* strains (data not shown). Fecal shedding data of *S. typhimurium* over the 12-d experimental period are presented in Figure 1. Data are presented by day, although there was no treatment × day interaction ($P > 0.05$). Populations of *S. typhimurium* ranged from 10^1 to 10^4 cfu/g of feces throughout the experiment. Overall, *S. typhimurium* populations tended to decrease by d 4, showed a slight increase on d 5 and 6, and then dropped to low levels by d 12. When examined across days, ionophore feeding had no effect ($P > 0.05$) on fecal shedding of *S. typhimurium*.

Luminal contents from the rumen, cecum, and rectum contained similar ($P > 0.05$) populations of *S. typhi-*

Table 2. Lumen contents and tissue samples positive for *S. typhimurium* and *E. coli* in sheep experimentally infected with *S. typhimurium* and fed a control (CON) diet or diets containing monensin (MON), bambermycin (BBM), or laidlomycin propionate (LP) (Exp. 1)

	Treatment				
Item	CON	MON	BBM	LP	SEM
S. typhimurium[a]					
Rumen	0.52	2.15	1.72	2.50	0.54
Cecum	0.28	0.88	0.33	1.45	0.42
Rectum	0	0	1.05	0.90	0.43
E. coli[b]					
Cecum	4.38	5.08	3.06	6.19	0.84
Rectum	4.65	5.27	3.76	6.21	0.62
No. positive for *S. typhimurium*[b]					
Rumen	2/4	3/4	4/4	3/4	
Cecum	3/4	3/4	4/4	3/4	
Rectum	1/4	2/4	3/4	2/4	
Lymph node	3/4	3/4	3/4	3/4	

[a]Measured as cfu (log_{10}) per gram of lumen content.
[b]Tissue samples positive after a 24-h enrichment.

murium, although CON animals had numerically lower counts compared with animals fed ionophores (Table 2). Tissue samples from the rumen, cecum, rectum, and lymph nodes were nearly all *Salmonella* positive after a 24-h enrichment, however there was not a treatment effect ($P > 0.05$). Contents from the cecum and rectum were positive for generic *E. coli*, but no treatment differences were noted ($P > 0.05$; Table 2).

The number of lambs per treatment with scours and the number positive for *S. typhimurium* after enrichment are presented in Table 3. The incidence of scours was similar among sheep on the CON, BBM, and LP diets, but was lower (less than half) in MON-fed animals ($P < 0.05$). When combined over the entire experimental period, the number of sheep shedding *S. typhimurium* was numerically, but not significantly, higher in all treated groups compared with CON animals. Ionophore treatment had no affect on antimicrobial susceptibility patterns of *S. typhimurium* isolates at d 1, 6, or 12 of the experimental period (data not shown).

Experiment 2. Sheep were examined the day prior to inoculation and found to be negative for *E. coli* O157:H7 populations capable of growth on novobiocin/naladixic acid supplemented agar, negative for *Salmonella*, and positive for generic *E. coli* (data not shown). Fecal shedding of *E. coli* O157:H7 showed no treatment × day interactions ($P > 0.05$). Overall, a steady decline in *E. coli* O157:H7 populations was observed through d 10,

Table 3. Incidence of scours (SC) and fecal shedding of *S. typhimurium* (ST) in experimentally infected sheep fed a control diet (CON) or diets containing monensin (MON), bambermycin (BBM), or laidlomycin propionate (LP) (Exp. 1)[a]

	Treatment[a]							
	CON		MON		BBM		LP	
Day	SC	ST	SC	ST	SC	ST	SC	ST
1	2	3	4	4	4	4	4	4
2	2	3	1	2	2	2	0	3
3	2	3	1	4	3	3	2	2
4	2	3	2	4	3	2	2	3
5	2	3	0	4	1	2	2	3
6	2	3	0	3	1	3	1	3
7	1	2	0	3	1	4	1	4
8	1	1	0	2	3	3	2	3
9	2	1	1	1	1	1	2	2
10	1	1	0	2	0	3	1	1
11	1	1	0	1	1	1	1	2
12	1	1	0	2	1	3	1	2
Total	19	25	8	32	21	31	19	31
% of CON	—	—	42	128	110	124	100	124

[a]n = 4 lambs per treatment.

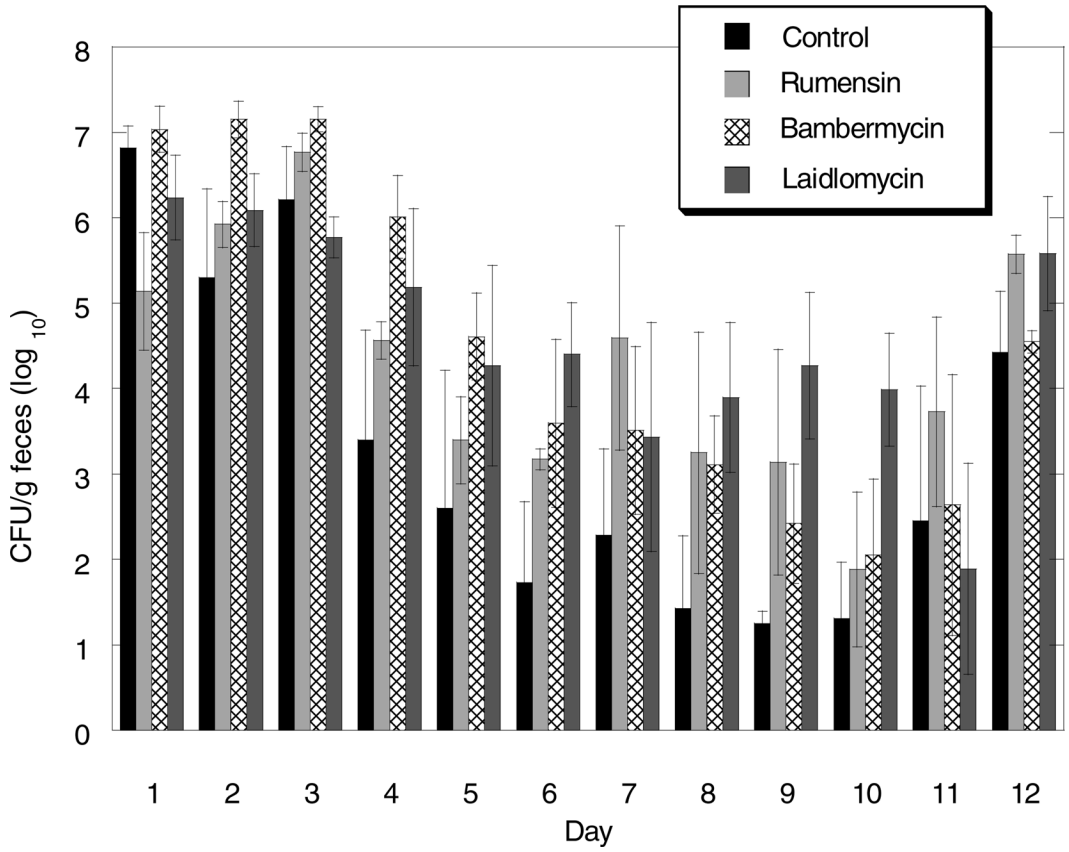

Figure 2. Fecal shedding (cfu/g feces [$\log_{10}$]) of *E. coli* O157:H7 in experimentally infected sheep fed a control diet or diets containing monensin, bambermycin, or laidlomycin propionate.

after which bacterial numbers began to increase over the last 2 d of the experiment (Figure 2). Ionophore treatment had no affect ($P < 0.05$) on fecal shedding compared with controls when data was pooled across days (3.27 vs. 4.26, 4.49, and 4.58 cfu ($\log_{10}$)/g feces for CON, MON, BBM, and LP treatments, respectively).

Overall, bacterial populations recovered from lumen contents of the rumen, cecum, and rectum were low with no differences ($P > 0.05$) observed among treatments (Table 4). *Salmonella* was not recovered from any of the gut samples. Tissue samples enriched for 24 h resulted in at least one *E. coli* O157:H7-positive tissue sample per treatment; however, no significant differences among treatments were noted (Table 4). The total number of sheep shedding *E. coli* O157:H7 over the 12-d experimental period was numerically higher ($P > 0.05$) for ionophore treatments when compared with control animals (Table 5). Antimicrobial susceptibility was generally unaffected by ionophore treatment at any of the sampling times examined (data not shown). However, the number of isolates resistant to streptomycin appeared to be lower in BBM compared with CON treatments (one vs. three isolates), and collectively, the *E. coli* isolates from the BBM treatment showed resistance to seven antibiotics compared with four antibiotics for the CON treatment; however, these differences were not significant.

Discussion

Ruminant animals are asymptomatic carriers of *E. coli* O157:H7 and other enterohemorrhagic *E. coli* (Rasmussen et al., 1993; Bielaszewska et al., 2000; Cornick et al., 2000), with the majority of human outbreaks linked to contact with ruminant animals or to products derived from them (Gage, 2001). Sheep, like cattle, are naturally colonized by *E. coli* O157:H7 in a transient and seasonal manner (Chapman et al., 1996; Kudva et al., 1997a,b; Johnsen et al., 2001) and have been documented with populations of *E. coli* O157:H7 similar to those found in cattle (Zschock et al., 2000). Although the number of human outbreaks of *E. coli* that are attributed to ovine rather than bovine sources are far fewer, sheep still serve as an effective and economical experimental model of *E. coli* O157:H7 colonization and infection (Kudva et al., 1995; 1997a; Cornick et al., 2000; Cookson et al., 2001).

Overall, we saw no effect of ionophore treatment on fecal shedding or gut populations of experimentally infected *E. coli* O157:H7 or *S. typhimurium*. Previous research, however, has yielded conflicting results. Garber et al. (1995) reported no association between fecal shedding of *E. coli* O157:H7 and ionophore use in dairy calves. Dargatz et al. (1997) reported no relationship between ionophore use and *E. coli* O157:H7 in

Table 4. Gut contents and tissue samples positive for *E. coli* O157:H7 and *Salmonella* spp. in sheep experimentally infected with *E. coli* O157:H7 and fed a control (CON) diet or diets containing monensin (MON), bambermycin (BMB), or laidlomycin propionate (LP) (Exp. 2)

	Treatment				
Item	CON	MON	BMB	LP	SEM
E. coli O157:H7[a]					
Rumen	0.58	0	0.63	0.30	0.29
Cecum	0	0	0	0	—
Rectum	0	0.28	0	0.30	0.20
Salmonella spp.[a]					
Cecum	0	0	0	0	0
Rectum	0	0	0	0	0
No. positive for *E. coli* O157:H7[b]					
Rumen	2/4	1/4	2/4	1/4	
Cecum	1/4	1/4	1/4	1/4	
Rectum	1/4	1/4	1/4	2/4	

[a]Measured as cfu (log_{10}) per gram of lumen content.
[b]Tissue samples positive after a 24-h enrichment.

feedlot cattle. In a survey of 100 feedlots in the United States, Losinger et al. (1997) found no difference in the number of fecal samples positive for *Salmonella* when ionophores were included in the diet. Rather, the prevalence of *E. coli* O157 was higher in dairy herds that used monensin, lasalocid, and/or decoquinate in their heifer rations compared with herds not using these additives (Herriott et al., 1998).

In support of our findings, Dealy and Moeller (1977) reported that calves supplemented with BBM in their feed had similar intestinal *E. coli* populations compared to control calves. However, these same authors reported that BBM decreased the percentage of *E. coli* resistant to streptomycin and oxytetracycline and the percentage of *E. coli* multiply resistant to two and three antibiotics. Sokol et al. (1973) demonstrated that feeding low levels of BBM to swine reduced tetracycline resistance in *E. coli*. Similar experiments showed that BBM reduced the number of *E. coli* resistant to streptomycin and sulfonamides (Federic and Sokol, 1973). Our research tends to agree with these reports. The number of isolates resistant to streptomycin appeared to be less in BBM compared to CON treatments (one vs. three isolates). Additionally, *E. coli* isolates from the BBM treatment showed resistance to seven antibiotics compared to four antibiotics for the CON treatment. The reason these observations were not statistically different may be related to the small sample size since we examined only one isolate per sheep, giving us a total of four isolates per treatment. However, the current concern over the subtherapeutic use of antibiotics in livestock production and the hypothesized connection to an in-

Table 5. Fecal shedding (number of positives by day) of *E. coli* O157:H7 in experimentally infected sheep fed a control (CON) diet or diets containing monensin (MON), bambermycin (BBM), or laidlomycin propionate (LP) (Exp. 2)

	Treatment[a]			
Day	CON	MON	BBM	LP
1	4	4	4	4
2	4	4	4	4
3	4	4	4	4
4	4	4	4	4
5	3	4	4	4
6	3	4	4	4
7	3	4	4	4
8	2	4	4	4
9	4	4	4	4
10	3	3	3	4
11	2	4	2	2
12	4	4	4	4
Total	40	47	46	46
% of CON	—	117	115	115

[a]n = 4 lambs per treatment.

crease in antimicrobial-resistant pathogens isolated from humans (Cohen and Tauxe, 1986) substantiates the importance and the need for continued research in this area.

The use of BBM-supplemented feed reduced the duration and prevalence of *S. typhimurium* shedding in experimentally infected calves (Dealy and Moeller, 1977). Furthermore, these authors reported that feeding BBM reduced the number of *Salmonella* resistant to streptomycin, ampicillin and oxytetracycline. Feeding swine BBM, likewise reduced the duration and prevalence of *Salmonella* shedding and decreased the number of *Salmonella* resistant to ampicillin, streptomycin, triple sulfa, and tetracycline (Dealy and Moeller, 1976). *Salmonella* recovery from various tissues was lower in pigs fed BBM compared to control animals leading these authors to conclude that BBM did not increase the carrier state of *Salmonella* in pigs. In contrast, we did not observe any difference in the duration or levels of *Salmonella* shedding nor were any differences noted in antimicrobial susceptibility patterns. We also observed no differences in the number of tissue samples positive for *Salmonella*, indicating BBM neither increased nor decreased the carrier state of *Salmonella* in our study. The effect of MON on decreasing the incidence of scours is interesting but difficult to explain considering no treatment differences were observed in the number of positive GIT tissue samples, lumen content concentrations or fecal shedding in these same animals.

Research results examining the effects of ionophores on *E. coli* and *Salmonella* are conflicting and highlight the complexity of the ruminant animal. While the results of our research typically agree with most reports concerning MON, we did not see the effects of BBM on these pathogens reported by others. Differences may be due to animal species, duration of experiment, BBM concentration fed, or other variables. We conducted a power analysis on our data to determine if more animals per treatment were needed. Results from Exp. 1 indicate that because so little difference was seen between treatments, thousands of animals would be needed (0.80 power). In Exp. 2, power analysis indicated (0.08 power) that tripling the number of animals per treatment may have produced significant differences. Because this was a terminal study, we used a small number of animals to look for major differences in shedding patterns. Increasing animal numbers as the power analysis indicates in Exp. 2, may have detected small significant differences (one-half log) in shedding, however these differences would be meaningless in modern livestock production. It should be noted that although animals were individually penned, there was the chance for horizontal transmission to occur among animals. To support this, fecal shedding of *E. coli* in Exp. 1 steadily decreased over time until about d 10, at which time numbers began to increase. However, if examined from a practical livestock production standpoint, horizontal transmission would be the norm rather than the exception.

Implications

The overall effect of short-term feeding of ionophores on *Salmonella* and *E. coli* O157:H7 in experimentally infected sheep was negligible. The effects of long-term ionophore feeding (i.e., dairy heifers and feedlot cattle) on gut populations and shedding of food-borne pathogens in ruminants warrants further research. Disseminating the factors involved in the carriage and shedding of food-borne pathogens in ruminants is a complex, but necessary task, as long as there is opportunity for contamination of our food supply. Incorporating knowledge from this and other research, along with pre- and post-harvest treatment methodologies may help to improve the safety of ruminant derived foods.

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